



## TA-MUC1 epitope in non-small cell lung cancer

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### KEYWORDS

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**Summary** MUC1 (CD227), an established tumor marker, is expressed on glandular epithelia and on epithelial tumors. Tumor MUC1 differs from normal MUC1 by modified glycan side chains. Recently, a novel carbohydrate-induced conformational tumor-associated MUC1 epitope (TA-MUC1) was described, whose clinical relevance in lung cancer is not known. Eighty-five paraffin embedded tissue sections of non-small cell lung cancer (NSCLC) patients (73% male; mean age  $64 \pm 9$  years) were stained with the monoclonal antibody PankoMab (against TA-MUC1) and compared with the established antibodies E29 and 214D4 regarding prognostic relevance. TA-MUC1 is virtually absent in bronchial epithelium. As shown by multivariate analysis, only staining with PankoMab, but not with E29 or 214D4, was correlated with patients' survival ( $p = 0.029$ ). Moreover, when regarding interactions of MUC1 antibody staining results and clinico-pathological parameters, patients with lymph node metastasis lacking PankoMab staining were attributed the highest risk by far (Hazard ratio = 4.6, 95% CI: 2.1–9.7,  $p = 0.000$ ). In summary, the presence of TA-MUC1 is a favorable prognostic factor in this cohort of NSCLC patients, in particular if lymph node metastases are present. This is in contrast to the results for E29 and 214D4, which recognize less or not glycosylation dependent epitopes. As this is the first report on a well-defined MUC1 epitope associated with improved survival in NSCLC, a more differentiated view on MUC1 may be mandatory.

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## 1. Introduction

Lung cancer has become the leading cause of cancer-related death in industrial countries [1]. In 2002, about 1.18 million people died of lung cancer worldwide [2]. With 1.35 million cases newly diagnosed each year, lung cancer is also the most frequent newly diagnosed major cancer [2,3]. In developed countries the 5-year survival rate averages around 10–15% and has not significantly improved in the last 30 years [4]. It is also expected that lung cancer mortality rates are going to worsen in the years to come [5]. This underlines the importance of improving both diagnostics and therapy by getting a better understanding of lung cancer biology.

MUC1 (CD227) is a transmembrane glycoprotein that is expressed on normal glandular epithelia and on epithelial tumors [6,7]. It consists of a core protein with an intracellular tail, a transmembrane region and a highly *O*-glycosylated extracellular region [8]. The extracellular domain exceeds other glycoproteins of the glycocalyx in length [9] and consists of a variable number of tandem repeats including serines and threonines to which glycan side chains are attached [8,10]. The variable tandem repeat region (VNTR) includes 20–120 repeats of a 20 amino acid sequence [8]. The sequence itself can also be subjected to a polymorphism [11]. Several alterations of MUC1 expression in malignancy are known. In general, MUC1 in tumors is (i) overexpressed [12,13], (ii) no longer restricted to polar (apical) localization [7,9], and (iii) differs from normal MUC1 by aberrant glycosylation, i.e., truncated, shorter glycan side chains [10,14]. The altered MUC1 on tumor cells is supposed to have an anti-adhesive effect, mediated by a net negative charge of the plasma membrane provided by extra sialic acid residues [15] and steric hindrance [15,16], which interferes with E-cadherin [9], contributing to nodal and distant metastasis [17].

There are a large variety of monoclonal antibodies (mAbs) against MUC1 available, which recognize different epitopes. Several of these mAbs are even regarded as Pan-MUC1 [18] antibodies. Varying antibody features (Pan-MUC1 vs. differentiation-dependency, glycosylation dependent vs. independent), the plethora of studies using (Pan-)MUC1-antibodies described the expression of MUC1 as a negative prognostic factor in malignancy (Table 1). With regard to non-small cell lung cancer (NSCLC), the presence of MUC1 has been described as a poor prognostic marker for survival as well [19–21].

In a comparison of multiple antibodies towards the immunodominant sequence of MUC1 (including those from the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1 [22]) it was found that antibodies generated against tumor MUC1 (in contrast to antibodies generated against non-tumor MUC1) preferably recognize a conformational motif, generated by a specific glycosylation [18,23,24], which is considered to be a highly tumor-associated MUC1 (TA-MUC1) epitope [25,26]. Recently, an antibody named PankoMab has been developed to bind to this tumor-associated conformational epitope with high specificity [25].

In a clinical setting the antibody for TA-MUC1 (i.e., PankoMab) has not been tested yet. Here, we analyzed the specificity of PankoMab and the prognostic relevance

of the tumor-associated PankoMab epitope TA-MUC1 regarding patients' overall survival. In addition, we compared our findings to immunohistochemistry results using common Pan-MUC1 antibodies [18] (clones 214D4 [9,19] and E29 [27], respectively). We describe, for the first time, that TA-MUC1 (in contrast to the epitopes of E29 and 214D4 antibodies) is not ubiquitously expressed in NSCLC and that its presence is an independent prognostic factor for improved survival after surgical resection in NSCLC patients with lymph node metastasis.

## 2. Methods

### 2.1. Study population

Out of patients admitted into the Pulmonary Division of Johannes Gutenberg University Medical Center (Mainz, Germany) we identified those who were newly diagnosed with NSCLC, underwent surgical resection and had a complete follow-up. Eighty-five NSCLC patients diagnosed between 1995 and 2003 with resection of the primary lung lesion were included. The study was performed compliant to the rules and regulations of the state ethics committee, all subjects gave written consent. Medical records were reviewed for clinical data including primary symptoms, laboratory parameters (i.e., calcium, GOT, ALP, LDH, creatinine, established lung tumor markers (carcinoembryonic antigen, neuron-specific enolase and cytokeratin fragment 19), blood count, respiratory function, ECOG performance status, surgical and pathological classification, and follow-up data). Clinical TNM staging (including clinical examination, CT scans, sonography, endoscopy, MRI, bone scan) was performed according to IUCC/AJCC recommendations [28]. To determine a definite tumor stage [29], post-surgical pathological examination was included (Table 2).

The study population included patients of IUCC stages [28] IA–IIIB and various treatment plans supplementing lung surgery. In more detail, 59% of patients were defined as stage I, 31% as stage II, and 11% as stage III (Table 2). Seventy-four patients (87%) underwent surgical treatment of the primary pulmonary lesion only. Six patients (7%) received radio-therapy after surgery, one patient (1%) received adjuvant chemotherapy. One patient (1%) received neoadjuvant chemotherapy; three patients (4%) received neoadjuvant combined radio-chemotherapy. All chemotherapies were platinum-based. In summary, lung surgery was augmented by multimodal therapy in 11 patients (13%) using radiation, chemotherapy, or both.

According to the WHO classification of lung tumors [30], the primary pulmonary lesion was classified as squamous cell carcinoma (SCC) in 34 patients (40%), 39 patients (46%) had adenocarcinoma (including six bronchioloalveolar carcinomas (BAC)), and five (6%) had adenosquamous carcinoma. Five patients had large cell carcinoma (6%). Two (2%) tumors were non-small cell lung cancer without further classification (Table 2).

All patients had follow-up visits on a regular basis. Systematic restaging was performed after 3, 6, 12, 18, 24, 36, 48, etc. months or earlier if necessary. Restaging included clinical examination, chest X-ray, abdominal ultrasound scan and blood tests. CT scans were performed if progression

Table 1 Comparison of several MUC1 antibodies

Antibody	Epitope <sup>a</sup>	GD <sup>b</sup>	Cancer	Study results <sup>c</sup>	Published by
B27.29 <sup>d</sup>	PDTRPAP	GD-2	Breast cancer	Low grade carcinoma, lymph node metastasis	[40]
Ma552	GVTSAPDTRPAP	GD-2	Colorectal carcinoma	Depth of tumor invasion, lymph node metastasis	[41]
E29 <sup>d</sup>	APDTRP	GD-2	Gastric cancer	Advanced stage and liver metastasis	[27]
HMFG1	APDTR	GI	Renal cell carcinoma	Tumor size and distant metastasis	[42]
BC2 <sup>d</sup>	APDTR	GI	Breast cancer	Poor survival, shorter PFS	[43]
214D4 <sup>d</sup>	PDTR	GI	Lung cancer	Poor prognosis	[19]
DF3	APDTRPAP	n.a. <sup>e</sup>	Lung cancer	Poor prognosis	[20]
PankoMab	PDT(*)RP	GD-A	Lung cancer	Better prognosis, especially in patients with lymph node metastasis	current study

GI: glycosylation independent (ratio 0.8–1.2), GD-2: moderately glycosylation dependent (ratio 1.3–5.9), GD-1: strongly glycosylation dependent (ratio  $\geq 6$ ), GD-A: absolutely glycosylation dependent (ratio  $\geq 20$ ).

<sup>a</sup> Glycosylated with Thomsen–Friedenreich antigen (TF) or precursor (Tn).

<sup>b</sup> Amino acid sequence.

<sup>c</sup> Glycosylation dependency (absorbance ratio glycosylated: unglycosylated) according to [23–25].

<sup>d</sup> Summary of study result, association of MUC1 expression.

<sup>e</sup> Considered as Pan-MUC1 antibody according to [18].

<sup>f</sup> Not available.

was suspected. Survival time and progression-free survival time (PFS) were calculated from the date of histological diagnosis to death, progressive disease, or last contact, respectively. In the latter case, the survival time was regarded as censored. Sixty-two patients (73%) developed progressive disease. Among them, 16 patients (19%) were further treated with chemotherapy, three (4%) were treated with radiochemotherapy, radiotherapy was applied in twelve cases (15%), four patients (5%) were surgically resected for a second time, and two patients (2%) received radiotherapy after surgery of the progressive lesion. Twenty-seven (42%) patients received best supportive care.

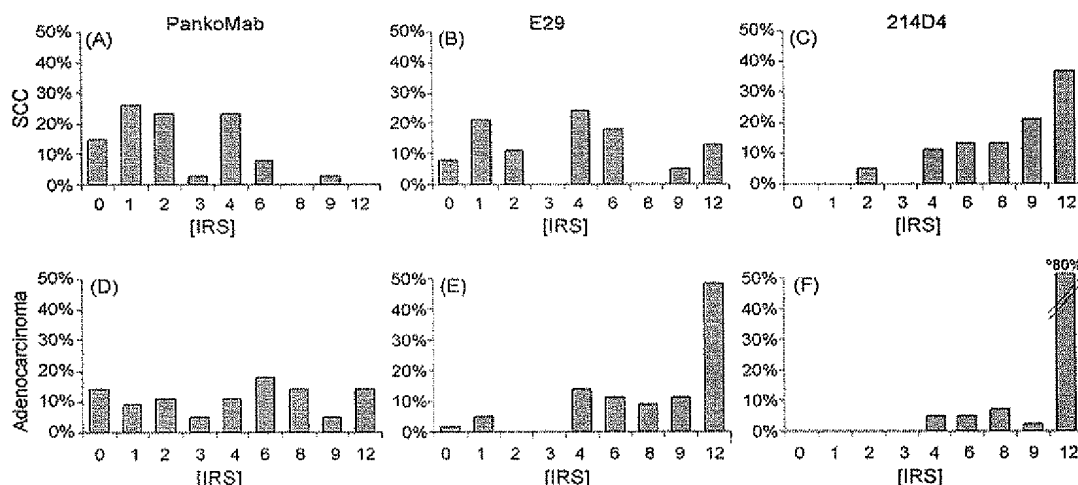
## 2.2. Immunohistochemistry

After surgery fresh tumor tissue specimens were immediately formalin-fixed and afterwards embedded in buffered paraffin. After removal of paraffin, the slides were heated in pH 9 buffer (Target Retrieval Solution, Dako, Hamburg, Germany). All slides were stained simultaneously using a computer controlled autostainer (Dako TechMate 500) and the Dako EnVision-System [31]. Unspecific tissue peroxidases were blocked by H<sub>2</sub>O<sub>2</sub> (POD-Block Dako EnVision) then the slides were incubated for 60 min with the monoclonal primary MUC1 antibodies (PankoMab [25] dilution 1:10 (Glycotope, Berlin, Germany), E29 [27] 1:200 (Dako), 214D4 [19] 1:100 (Upstate, Lake Placid, NY)). The low dilution of PankoMab was chosen to demonstrate minimal unspecific binding of this mAb in non-malignant tissue. A secondary anti-mouse-antibody linked to peroxidase by dextrin chains (Dako EnVision) was added. Sections were stained with DAB-substrate (Dako), counterstained by hematoxylin solution (Dako) and finally covered with Entellan (Merck, Darmstadt, Germany). During the staining procedure with E29 two slides (2%) were damaged and could not be evaluated later. Regarding 214D4, five slides (6%) could not be evaluated.

The slides were classified by three investigators (F.B., K.S. and A.K.) according to Remmele's Immunoreactive Score (IRS) [32]. For the evaluation of the IRS, only tumor cells were taken into account. Percentage of positive cells (0% = 0 point, 1–10% = 1 point, 11–50% = 2 points, 51–80% = 3 points, 81–100% = 4 points), and staining intensity (weak = 1, moderate = 2, strong = 3) were evaluated and multiplied. An IRS  $\geq 3$  was considered as positive (Fig. 1).

## 2.3. Statistical analysis

Standard descriptive statistics such as frequencies, mean, standard deviation, median and standard error were calculated to describe the study population. One of the principal aims of this study was to show the prognostic value of the carbohydrate-induced conformational tumor epitope (TA-MUC1) on patients' survival. Therefore, results of immunohistochemistry using PankoMab were compared to antibody staining using E29 and 214D4. In addition to the comparative analysis of the staining results, the clinicopathological correlations and dependencies for antigenetic expressions regarding sex, age, performance status, pT, pN, stage, and histopathological criteria were assessed using Fisher's exact test. The prognostic analysis was carried out by several multivariable analyses using Cox proportional hazards regression. Features considered as potential explanatory factors (reference category underlined) were sex (male vs. female), age (as a continuous variable), performance status (ECOG 0 vs. ECOG 1 and 2), body-mass index (BMI, as a continuous variable), stage (1 vs. 2 vs. 3), lymph node metastasis (N0 vs. N1 and N2), histological tumor type (squamous cell carcinoma vs. other NSCLC, adenocarcinoma vs. other NSCLC, large cell carcinoma vs. other NSCLC), multimodal treatment (lung surgery only vs. adjuvant/neoadjuvant therapy), and antibody staining using PankoMab, E29 and 214D4 (negative vs. positive).



**Fig. 1** Lung cancer immunohistochemistry. Staining frequencies with respect to the Immunoreactive Score (IRS) are shown for the monoclonal antibodies PankoMab (A and D), E29 (B and E), and 214D4 (C and F). Between the two main entities, adenocarcinoma (D–F) was positively stained (red columns) with PankoMab, E29, and 214D4 in a higher frequency (66%, 93%, and 100%) than squamous cell carcinoma (A–C: 36%, 60% and 95%). Staining frequency and intensity increased from PankoMab (A and D: longest epitope and highest glycosylation dependency) over E29 (B and E) to 214D4 (C and F: shortest epitope and no glycosylation dependency, compare Table 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

To analyze the prognostic value of the potential explanatory factors, a Cox proportional hazards model was applied using a forward stepwise selection (inclusion criteria:  $p$ -value of the Score test  $\leq 0.05$ , exclusion criterion:  $p$ -value of the likelihood ratio test  $\geq 0.1$ ). Eight patients (9%) had at least one missing value; therefore they had to be omitted from this analysis. Similarly, a Cox regression analysis with a stepwise backward selection (inclusion criteria:  $p$ -value of the Score test  $\leq 0.05$ , exclusion criterion:  $p$ -value of the likelihood ratio test  $\geq 0.1$ ) was performed. To analyze more cases, further Cox regression analysis with the factors selected by the first and second Cox regression model was performed using forward and backward selection models. Eighty-one patients (95%) were included in this analysis. Finally all factors were supplemented by reasonable interactions for a forward selection Cox regression model which was confirmed by a forward and backward selection model as already described. Interactions tested were (squamous cell carcinoma, adenocarcinoma, lymph node metastasis)  $\times$  (PankoMab, E29, 214D4).

All analysis were performed using SPSS® 13 and 14 software (SPSS GmbH, München, Germany) and were regarded as explorative, therefore a global or local level of significance was not determined.

### 3. Results

#### 3.1. Descriptive statistics

Median survival time of the patients observed in this study was  $1444 \pm 204$  days (min. 40, max. 3449 days, Table 2), and 29 of 85 cases (34%) were censored with a median follow up time of 4.4 years. Median progression-free survival time

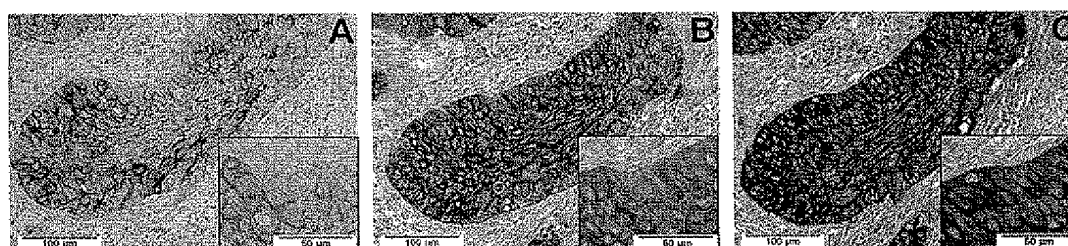
was  $925 \pm 127$  days (min. 21, max. 3449 days), 23 of 85 cases were censored (27%).

#### 3.2. Immunohistochemistry in lung tumors

To analyze staining results, an established scoring system, the Immunoreactive Score according to Remmele and Stegner [32], suitable for clinical routine, was used in our study. Positive staining with PankoMab was observed in malignancy in 52% of all cases (44/85 cases). PankoMab positive slides showed clear cytoplasmatic staining and/or membrane staining with an IRS value of at least 3 (Fig. 1A and D; Fig. 2A). A comparison of apical and depolarized MUC1 staining pattern as described by Guddo et al. [19] was not applicable, since virtually none of the samples had an apical tumor staining only. As for E29, positive staining was observed in malignancy in 77% of all cases (65/83 cases, two were not evaluable). The majority of the E29 positive samples (36/65 cases, 55%) showed a highly positive staining as indicated by an IRS  $\geq 9$  (Fig. 1B and E; Fig. 2B). As for 214D4, positive staining was observed in almost all cases (95%, 76/80 cases, five were not evaluable), showing very high IRS values (Fig. 1C and F; Fig. 2C).

Positive staining with E29 was highly associated with both PankoMab and 214D4 positive staining (Fisher's exact test  $p < 0.001$ ,  $p = 0.001$ , respectively), however, PankoMab staining was not associated with 214D4.

Since the paraffin blocks were collected over a time period of more than 8 years, block age and staining results were compared using the Kruskal–Wallis  $H$ -test to detect a potential change in antigen expression over time. For PankoMab, E29 and 214D4 antibody staining, no significance concerning the age of the collected tumor samples could be shown ( $p = 0.88$ ,  $0.37$ , and  $0.831$ , respectively).



**Fig. 2** Staining characteristics of PankoMab, E29, and 214D4. Typical staining patterns are representatively shown in squamous cell carcinoma (A–C). Regarding tumor cells, all antibodies showed either cytoplasmatic staining, or a combination of cytoplasmatic and membranous staining. IRS is 9 (A) and 12 (B and C). The percentage of combined staining rose from PankoMab over E29 to 214D4 (A–C). In this representative sample of an adenocarcinoma IRS of tumor cells (not mapped) is 12 for all antibodies. Images are 20 $\times$ , inlays are 40 $\times$ , bar = 100  $\mu$ m or 50  $\mu$ m, respectively.

### 3.3. Immunohistochemistry regarding histological tumor types

Different frequencies of PankoMab positive staining could be observed depending on histology. Between the two

main entities, adenocarcinoma had a higher frequency for PankoMab positive, E29 positive, and 214D4 positive staining (66%, 93%, and 100%; Fig. 1D–F) than squamous cell carcinoma (36%, 60% and 95%; Fig. 1A–C). Regarding each histological entity, median IRS scores rose from PankoMab over E29 to 214D4 antibody staining (median IRS values for squamous cell carcinoma and adenocarcinoma were 2, 4, and 9; and 5, 9, and 12, respectively Fig. 1A–F).

**Table 2** Baseline characteristics of the study population

Parameter	Patients evaluated (n = 85) % <sup>a</sup>	
Age (years)	64 $\pm$ 9 <sup>b</sup>	
Sex	62 males	73
Smoker or former smoker	77	94
Performance status		
ECOG 0	45	56
ECOG 1	35	43
ECOG 2	1	1
pT <sup>c,d</sup>		
T1	30	35
T2	47	55
T3	7	8
T4	1	1
pN <sup>c,d</sup>		
N0	55	65
N1	24	28
N2	6	7
Stage <sup>c,d</sup>		
I	50	59
II	26	31
III	9	11
Histological tumor type		
Squamous cell carcinoma	34	40
Adenocarcinoma and BAC	39	46
Adenosquamous carcinoma	5	6
Large cell carcinoma	5	6
Unspecified NSCLC	2	2
Survival time (days)	1444 $\pm$ 204 <sup>e</sup>	

<sup>a</sup> Percent of non-missing values.

<sup>b</sup> Mean  $\pm$  S.D.

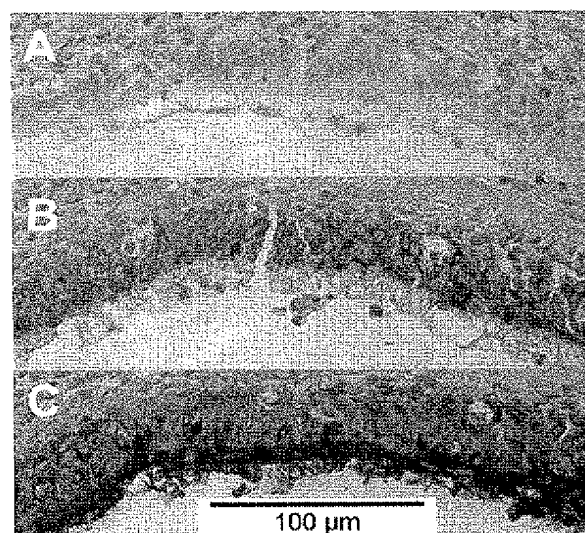
<sup>c</sup> Based on pathological assessment.

<sup>d</sup> Of note: four patients have pT3N0, one has pT4N0 disease.

<sup>e</sup> Median  $\pm$  S.D.

### 3.4. Immunohistochemistry in non-malignant tissue

Using three different antibodies against MUC1, we observed distinct varieties in the reactivity with non-malignant tissue. Regarding bronchial epithelium and epithelial cells, we did not observe staining of apical cilia for PankoMab (Fig. 3A),



**Fig. 3** Immunohistochemistry of bronchial epithelium. Using three different antibodies against MUC1 (A–C), staining of bronchial epithelium and epithelial cells displayed distinct characteristics. Using PankoMab (A), absent to faint staining of apical cilia for PankoMab was observed, which was more intense for E 29 (B) and 214D4 (C), including epithelial cells. Images are 20 $\times$ , bar = 100  $\mu$ m.

but for E 29 and 214D4, including epithelial cells (Fig. 3B and C). In tumor stroma, we found no reactivity with PankoMab, and occasionally faint staining with E 29 and 214D4 (data not shown). All tested antibodies reacted to a certain extent with pneumocytes (data not shown).

### 3.5. Associations of immunohistochemistry with clinicopathological parameters

Using crosstabs (two-sided Fisher's exact test and Chi-square test), we found positive staining results of PankoMab and E29 to be associated with adenocarcinoma ( $p=0.009$  and  $0.001$ , respectively). PankoMab was associated with stage III ( $p=0.03$ ) and N2-status ( $p=0.006$ ), but not with the incidence of lymph node metastasis (i.e., N0 vs. N1-2). E29 was not associated with stage, pN or the incidence of lymph node metastasis. 214D4 reactivity was not associated with any clinicopathological parameter. No other associations of immunohistochemistry with clinical parameters and histological factors (i.e., sex, ECOG, pT, large cell carcinoma, progressive disease) could be observed for any of the three tested MUC1-antibodies. Of note, all never-smokers ( $n=5$ ) were PankoMab negative ( $p=0.055$ ).

### 3.6. Prognostic relevance of the carbohydrate-induced conformational epitope TA-MUC1

Since one of the principal aims of this study was to show the prognostic value of the carbohydrate-induced conformational tumor epitope TA-MUC1 on patients' survival, we used a Cox proportional hazards model for comparison with established prognostic factors and common MUC1 antibodies E29 and 214D4. The starting model revealed age, BMI, ECOG, lymph node metastasis, and positive PankoMab staining (=TA-MUC1 expression) as explanatory prognostic factors, confirmed by backward regression model analysis (Table 3). In a second step, a Cox regression model with previously accepted factors was performed. Only lymph node metastasis and PankoMab immunohistochemistry results were accepted in both forward and backward likelihood ratio model (Table 3). In summary, positive PankoMab staining was a positive prognostic factor, whereas the presence of lymph node metastasis indicated poorer prognosis in this multivariate analysis. However, in univariate Kaplan–Meier estimates for overall survival, an association was only observed for lymph node metastasis (median survival time  $1889 \pm 520$  days vs.  $817 \pm 244$ , log rank  $p=0.031$ , Fig. 3B), but not for PankoMab staining ( $p=0.158$ ; Fig. 3A).

To assess whether these two independent factors are still valid if interactions were considered, we included reasonable interactions of histological tumor type, nodal status, and antibody staining results (E29, 214D4, and PankoMab, respectively). Regarding all parameters and interactions, patients with lymph node metastasis lacking PankoMab staining were attributed the highest risk by far (Table 4). Kaplan–Meier estimates for overall survival highlight this finding, since this subgroup of patients had a reduced median survival time ( $681 \pm 52$  days, log rank  $p<0.001$ , all comparisons) in contrast to patients with PankoMab positive staining or without nodal disease (median survival times

$1599 \pm 355$ ,  $1610 \pm 213$ , and  $2422 \pm 909$  days) (Fig. 3C). Regarding progression-free survival, this association was less evident ( $p=0.053$ , Fig. 3D).

However, the according Cox proportional hazards model for progression-free survival showed that in addition to sex ( $p=0.000$ ) and BMI ( $p=0.005$ ), the interaction of PankoMab and lymph node metastasis ( $p=0.007$ ) was a strong predictor, but not PankoMab staining and lymph node metastasis alone (data not shown).

To preclude a confounding effect of adenocarcinoma (preferentially stained by PankoMab) on the results of the survival analysis, survival times of patients with lymph node metastasis with and without adenocarcinoma were compared and no effect was found (Kaplan–Meier log rank  $p=0.81$ ).

## 4. Discussion

The heterogeneity of non-small cell lung cancer, presenting in various entities and with different clinical behavior is a major challenge both for clinicians and for researchers. The therapy is often based on clinical stage, tumor morphology and performance status alone. Determining an optimal individualized treatment plan, however, needs a better understanding of the biology of lung cancer and new prognostic markers. Here, we describe the prognostic relevance of the carbohydrate-induced conformational MUC1 tumor epitope (TA-MUC1) recognized by PankoMab regarding patients' overall survival after surgical resection of the primary lung tumor. Moreover, we compared the PankoMab immunohistochemistry results with those obtained using common Pan-MUC1 antibodies (E29 and 214D4). By multivariate analysis, we demonstrated that only PankoMab, but not E29 and 214D4, define an epitope that is an independent prognostic factor favoring improved survival in resected NSCLC patients, especially in patients with nodal disease.

PankoMab is a novel anti-MUC1 antibody designed to bind to the carbohydrate-induced conformational epitope of MUC1 with very high glycosylation dependency [25]. PankoMab binds to the immunodominant region within the tandem repeats only when the T of the PDTRP sequence is glycosylated by either the carbohydrates TF (Thomsen–Friedenreich antigen) or Tn (TF precursor) [33], two tumor-associated carbohydrate antigens which might add to the improved tumor-specificity of PankoMab [25]. Furthermore, PankoMab is rapidly internalized, has ADCC mediated activity and has already been successfully toxin- and radio-labelled for targeted therapy as shown by in vitro studies [25].

Despite the fact that most normal epithelial cells express the MUC1 glycoprotein [10], the PankoMab epitope was detected only in 52% of all evaluable NSCLC patients (Fig. 1). PankoMab did virtually not stain on bronchial epithelium (Fig. 3A) compared to E29 (Fig. 3B) and 214D4 (Fig. 3C). Most adenocarcinomas (69%) were stained by PankoMab (Fig. 1D), while squamous cell carcinomas were stained with lower frequencies (36%, Fig. 1A).

Our main finding is that the presence of TA-MUC1 is a favorable prognostic factor regarding all patients (Table 3). Moreover, when looking at PankoMab staining in NSCLC patients with lymph node metastasis, the association with



**Table 3** Overall survival: explanatory prognostic factors in a Cox proportional hazards model

Starting model <sup>a</sup>	FWD <sup>b</sup>			BWD <sup>c</sup>		
	HR <sup>d</sup>	95% CI <sup>e</sup>	p <sup>f</sup>	HR	95% CI	p
Age <sup>g</sup>	1.05	1.01–1.08	0.008	1.05	1.01–1.08	0.008
BMI <sup>h</sup>	0.87	0.79–0.95	0.002	0.87	0.79–0.95	0.002
ECOG <sup>i</sup>	2.63	1.38–5.04	0.003	2.63	1.38–5.04	0.003
Lymph node metastasis <sup>j</sup>	4.12	2.05–8.28	0.000	4.12	2.05–8.28	0.000
PankoMab <sup>k</sup>	0.51	0.28–0.94	0.029	0.51	0.28–0.94	0.029
Final model	FWD			BWD		
	HR	95% CI	p	HR	95% CI	p
Age	Not accepted			1.04	1.00–1.07	0.027
BMI	Not accepted			0.90	0.82–0.97	0.008
ECOG	Not accepted			2.18	1.18–4.01	0.012
Lymph node metastasis	2.24	1.22–4.10	0.010	3.51	1.79–6.86	0.000
PankoMab	0.52	0.29–0.94	0.028	0.48	0.27–0.87	0.014

<sup>a</sup> Other factors not accepted by Cox regression model: sex, squamous cell carcinoma, adenocarcinoma, large cell carcinoma, stage, neoadjuvant/adjuvant treatment, E29 and 214D4 immunohistochemistry results.

<sup>b</sup> Forward likelihood ratio model.

<sup>c</sup> Backward likelihood ratio model.

<sup>d</sup> Hazard ratio: HR <1 suggests improved survival.

<sup>e</sup> Confidence interval.

<sup>f</sup> p-Value according to the likelihood ratio test.

<sup>g</sup> Per anno.

<sup>h</sup> Per kg/m<sup>2</sup>.

<sup>i</sup> ECOG = 0 vs. ECOG >0.

<sup>j</sup> N0 vs. N1-2.

<sup>k</sup> Immunohistochemistry: PankoMab negative vs. PankoMab positive.

improved survival is even stronger, as demonstrated by a Cox proportional hazards model ( $p=0.000$ , Table 4) and Kaplan–Meier charts (Fig. 4C.).

In contrast, Guddo et al. [19] showed an association of MUC1 expression (detected by 214D4) with poor prognosis in NSCLC and hypothesized that this is due to an interaction with E-cadherin-molecules. The antibody used by Guddo et

al. is considered to be a Pan-MUC1 antibody, which binds largely independent of MUC1 glycosylation (Table 1). Nagai et al. [20] found similar results using the differentiation dependent antibody DF3 [18]. In both studies a depolarized expression of MUC1 on tumor cells was assessed. Loss of polarization reflects a dedifferentiation of the tumor [34], usually reported to be a negative prognostic factor [35]. As

**Table 4** Overall survival: explanatory prognostic factors including reasonable interactions

Final model <sup>a</sup> including interactions <sup>b</sup>	FWD <sup>c</sup>			BWD <sup>d</sup>		
	HR <sup>e</sup>	95% CI <sup>f</sup>	p <sup>g</sup>	HR	95% CI	p
BMI <sup>h</sup>	Not accepted			0.94	0.87–1.01	0.078
ECOG <sup>i</sup>	1.91	1.06–3.43	0.030	2.02	1.12–3.65	0.019
Lymph node metastasis without PankoMab <sup>j</sup>	4.70	2.21–10.0	0.000	4.55	2.14–9.69	0.000

<sup>a</sup> Other factors not accepted by Cox regression model: sex, squamous cell carcinoma (SCC), adenocarcinoma, large cell carcinoma, stage, neoadjuvant/adjuvant treatment, E29 and 214D4 immunohistochemistry results. Interactions tested were (squamous cell carcinoma, adenocarcinoma, lymph node metastasis) × (PankoMab, E29, 214D4).

<sup>b</sup> Interactions not accepted by Cox regression model: lymph node metastasis with E29, lymph node metastasis with 214D4, PankoMab with SCC, E29 with SCC, 214D4 with SCC, PankoMab with adenocarcinoma, E29 with adenocarcinoma, 214D4 with adenocarcinoma.

<sup>c</sup> Forward likelihood ratio model.

<sup>d</sup> Backward likelihood ratio model.

<sup>e</sup> Hazard ratio: HR <1 suggests improved survival.

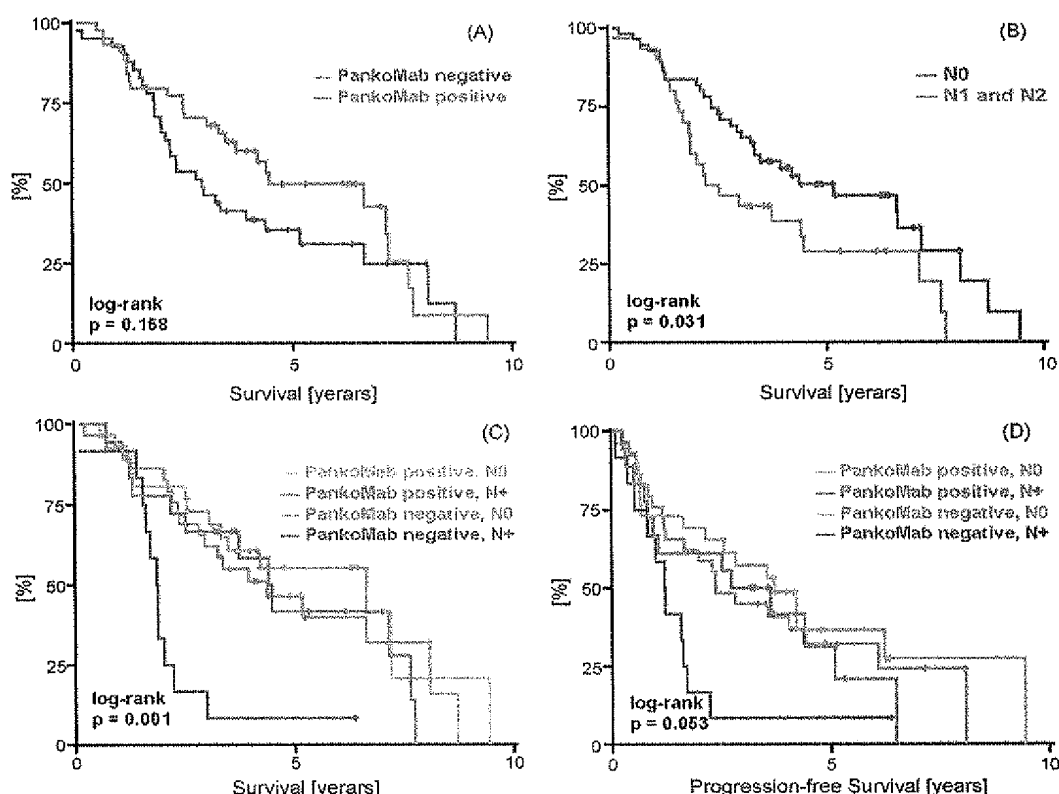
<sup>f</sup> Confidence interval.

<sup>g</sup> p-Value according to the likelihood ratio test.

<sup>h</sup> Per kg/m<sup>2</sup>.

<sup>i</sup> ECOG = 0 vs. ECOG >0.

<sup>j</sup> N0 and/or PankoMab positive vs. N1-2 and PankoMab negative.



**Fig. 4** Survival analysis. Kaplan–Meier estimates according to PankoMab immunohistochemistry (A) indicated a trend only, whereas nodal involvement was a better predictor of survival (B). However, in combination with nodal status, Kaplan–Meier estimates (C) highlighted that patients with lymph node metastasis lacking PankoMab staining were attributed the poorest survival (dark blue line). Of note, all patients in this group had N1 but not N2 disease. They had a more than twofold reduced median survival time ( $681 \pm 52$  days, log rank test  $p = 0.001$ ) in contrast to patients with PankoMab positive staining or without nodal disease ( $1599 \pm 355$  d (light blue),  $1610 \pm 213$  d (red),  $2422 \pm 909$  d (orange)). Multivariate analysis (Tables 3 and 4) confirmed the results. Regarding progression-free survival, the prognostic impact of the interaction between PankoMab staining and nodal status was more evident in multivariate analysis ( $p = 0.007$ ) than in univariate analysis (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nagai et al. reported an association of MUC1 expression with poorly differentiated cancer, we consider their results on the impact of Pan-MUC1 on survival to be partially confounded by tumor differentiation. We cannot completely rule out the possibility that our results contrasting former studies are at least partially influenced by technical issues, i.e., different antigen retrieval and immunostaining methods.

The majority of MUC1 antibodies used in studies on solid cancer were generated from non-tumor sources and are independent or moderately glycosylation-dependent [24,26]. Several of these mAbs can be regarded as Pan-MUC1 antibodies (Table 1). Despite varying antibodies, the plethora of studies described the expression of MUC1 as a negative prognostic factor in malignancy (Table 1). Our results (Tables 3 and 4) using the Pan-MUC1 antibodies 214D4 and E29 do not provide any data to object the multitude of previous papers reporting MUC1 as a negative prognostic factor in (solid) cancer. However, using PankoMab, there seems to be at least one epitope on MUC1 which is not ubiquitously

expressed in NSCLC and is an independent prognostic factor for improved survival after surgical resection in NSCLC patients with lymph node metastasis.

Because we found TA-MUC1 expression to be of most prognostic relevance in patients with lymph node metastasis, we assume an immunological role of TA-MUC1, being most obvious in advanced disease, when the immune system deals with tumor antigens in lymphatic organs. In general, glycosylation of MUC1 is critical for T-cell response [36] and antigen processing [36,37]. In contrast to glycosylated MUC1 without expression of the carbohydrate induced conformational epitope, TA-MUC1 might even stimulate T-cell response (S. Goletz, personal communication). There is also some work on humoral response, regarding the favorable effect of natural circulating MUC1 antibodies [38,39]. Therefore, we believe that the results of our study might be attributed to a special immunogenicity of TA-MUC1, which should be the focus of future studies.



## 5. Conclusion

TA-MUC1 is a tumor-associated epitope on the otherwise ubiquitous epithelial molecule MUC1 and is recognized by Panko-Mab. In tumor, the TA-MUC1 epitope seems to be much less frequent than Pan-MUC1 epitopes. The presence of TA-MUC1 is a favorable prognostic factor in this cohort of NSCLC patients, in particular if lymph node metastasis are present. As this is the first report on a carbohydrate induced conformational MUC1 epitope associated with improved survival in NSCLC, a more differentiated view on MUC1 may be mandatory. Underlying the impact of glycosylation on antigen processing by cells of the immune system, we hypothesize that there might be a special immunogenicity of TA-MUC1.

## 6. Conflict of interest

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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